Context-sensitive transposition of IS6110 in mycobacteria

Sue Wall, Kiran Ghanekar, Johnjoe McFadden and Jeremy W. Dale

Author for correspondence: Jeremy W. Dale. Tel: +44 1483 876484. Fax: +44 1483 300374.
e-mail: j.dale@surrey.ac.uk

Molecular Microbiology
Group, School of Biological
Sciences, University of
Surrey, Guildford GU2 5XH,
UK

The rational use of IS6110 fingerprinting for studies of the molecular epidemiology and evolution of *Mycobacterium tuberculosis* requires understanding of the dynamics of transposition. In laboratory model systems, it has been shown that transposition is context-sensitive, i.e. it is influenced by the nature of the site in which the insertion sequence is presented. Stimulation of transposition by activation of an adjacent promoter supports the hypothesis that transposition occurs more readily from transcriptionally active locations. In addition, it has been shown that transposition can be enhanced by the expression of the transposase *in trans*. These findings imply that the frequency of transposition will vary substantially between different strains of *M. tuberculosis*, and furthermore that a hitherto stable strain may develop more rapid variation due to transposition into an active site. The use of IS6110 fingerprinting for the analysis of longer-range relationships between *M. tuberculosis* isolates therefore needs to be interpreted with care.

Keywords: transposition, IS6110, mycobacteria, tuberculosis, epidemiology

INTRODUCTION

The insertion sequence IS6110 (also known as IS986) (McAdam *et al.*, 1990; Thierry *et al.*, 1990; Zainuddin & Dale, 1989) has become established as the standard tool for molecular epidemiology of tuberculosis, exploiting the multiple polymorphism exhibited by most clinical isolates of *Mycobacterium tuberculosis* (Hermans *et al.*, 1990). The discriminating ability of IS6110 depends on the rate of variation being high enough to generate diversity in epidemiologically unlinked isolates, and yet being low enough for related strains to exhibit essentially identical patterns. Despite this, little is known of the factors that influence the frequency of transposition, or of the rate of variation that occurs in different strains. This lack of knowledge is becoming increasingly significant as more emphasis is placed on macro-epidemiological and evolutionary analysis of families of strains with partially related banding patterns.

Although most isolates of *M. tuberculosis* contain multiple copies of IS6110 (typically 10–20 copies), a minority (largely confined to South- and South-East Asia) possess only a single copy (Fomukong *et al.*, 1994). Most strains of *Mycobacterium bovis*, and BCG vaccine strains, carry either one or two copies (Fomukong *et al.*, 1992). The original sequence data indicated a small

number of differences in the structure of the element in BCG (IS987) (Hermans *et al.*, 1991) and *M. tuberculosis* (IS6110, IS986) (McAdam *et al.*, 1990; Thierry *et al.*, 1990), and it was suggested (Dale, 1995) that these differences could account for the apparent lack of mobility in BCG, and by extension, also in *M. bovis* and single-copy strains of *M. tuberculosis*. It is now known that these apparent differences were due to sequencing errors and the element is identical in both single-copy and multi-copy strains (Dale *et al.*, 1998). It is conceivable (albeit unlikely) that BCG is inherently unable to support transposition of IS6110; a more likely hypothesis is that the mobility of the element is influenced by its context, for example by requiring an external promoter, and that the site of insertion in BCG (and other low-copy-number strains) renders the element immobile. The purpose of this paper is to examine these hypotheses.

METHODS

Bacterial strains and plasmids. The mycobacterial strains used were *Mycobacterium smegmatis* mc²155 (Snapper *et al.*, 1990) and *Mycobacterium bovis* BCG (Pasteur strain). Electroporation of mycobacteria was by previously described methods (Dellagostin *et al.*, 1993). *M. smegmatis* was grown in nutrient broth No. 2 (Oxoid) or on corresponding nutrient agar; for BCG, we used 7H9 broth (Difco) or 7H11 agar

Fig. 1. Structure of plasmids used. (a) Suicide plasmids based on pGEM3Zf(+). *IS*, IS6110 (PCR-amplified using the primer of Fomukong & Dale, 1993); *aph*, kanamycin-resistance gene; *T7*, T7 promoter; *P*, *Pvu*II. (b) Conditionally replicating (temperature-sensitive) plasmids. *oriM*, temperature-sensitive mycobacterial origin of replication from pCG59; *IS*, IS6110 (PCR-amplified using the primer of Ghanekar *et al.*, 1999); *aph*, kanamycin-resistance gene; *B*, *Bam*HI. These plasmids are based on pUC19 (*E. coli* origin of replication not shown). (c) Mycobacterial shuttle plasmid for expression of transposase. *oriM*, mycobacterial origin of replication from pYUB12; *P*_{*hsp60*}, the promoter fragment of the *hsp60* gene from BCG (comprising 383 bp 5' upstream sequence plus the first six codons of *hsp60*); *transposase*, *Bsp*EI fragment of IS6110 (containing the coding regions of the insertion sequence); *hyg*, hygromycin-resistance gene from plasmid pEP3; *P*, *Pvu*II. The base vector was pGEM3Zf(+) (Promega).

Fig. 2. Transposition in *M. smegmatis*. (a) pUS252 transformants: Southern blots of total DNA, digested with *PvuII*, probed with the right-hand fragment of IS6110. Lanes: 1–11, pUS252 transformants; 12, untransformed *M. smegmatis*; 13, pUS252. (b) pUS1846 clones: Southern blots of total DNA, digested with *BamHI*, probed with complete IS6110. Lanes: 1, untransformed *M. smegmatis*; 2, pUS1846; 3–16, clones from the papillation assay.

(Difco), in both cases supplemented with OADC (Difco). For transposition assays in *Escherichia coli*, we used strain JM109(DE3) (Promega), which carries an IPTG-inducible T7 RNA polymerase gene.

The plasmids used are shown in Fig. 1. The pUS25x series of suicide plasmids was based on pGEM3Zf(+) (Promega), while the pUS1800 series of conditionally replicating vectors was based on pUC19 with the addition of a 1.6 kb DNA fragment containing the origin of replication from the thermosensitive mycobacterial plasmid pCG59 (Guilhot *et al.*, 1992). In the pUS25x series, the IS6110 fragment was PCR-amplified using the primer of Fomukong & Dale (1993); the primers used for the pUS1800 series were described by Ghanekar *et al.* (1999). In both cases, cloned template derived from the bacteriophage lambda clone A3 (Zainuddin & Dale, 1989) was used. The shuttle plasmid pUS267 was constructed

using the 2.5 kb *HpaI*–*EcoRV* fragment of pYUB12 (Snapper *et al.*, 1990) containing a mycobacterial origin of replication, an *hsp60* promoter fragment from BCG (comprising 383 bp 5' upstream sequence plus the first six codons of *hsp60*) upstream from the *BspEI* fragment of IS6110 (containing the coding regions of the insertion sequence), and a hygromycin-resistance gene from plasmid pEP3 (Radford & Hodgson, 1991); the base vector was pGEM3Zf(+) (Promega).

For each series of plasmids, the nature and orientation of the inserts were confirmed by restriction digestion and sequence analysis.

Papillation assay. The papillation assay for transposition of IS6110 from the conditionally replicating plasmids in *M. smegmatis* was described in detail by Ghanekar *et al.* (1999). Transformants were grown in nutrient broth at the permissive temperature (30 °C) to a density of about 5×10^7 c.f.u. ml⁻¹.

Aliquots (200 µl) were spread on nutrient agar plates supplemented with kanamycin (30 µg ml⁻¹). The plates were incubated at 30 °C for 2–3 d until a lawn was just visible. They were then exposed to a microaerobic environment in a gas jar with a gas generation system (Campylobacter BR56; Oxoid) for 48 h before being removed, sealed and incubated in air at the restrictive temperature (39 °C). Papillae were scored after 14 d, and the frequency of papillae was estimated as a proportion of the total number of colonies on the plate.

Southern blotting and probes. Total DNA, digested with appropriate enzymes and electrophoresed through an agarose gel, was transferred to a positively charged nylon membrane (Boehringer Mannheim) and hybridized to digoxigenin-labelled probes. Chemiluminescent detection was performed according to the manufacturer's instructions (Boehringer Mannheim). For the pUS25x series of plasmids, the IS6110 probe consisted of the region to the right of the *PvuII* site generated by PCR with the primers INS-1 (Hermans *et al.*, 1990) and BX-2 (Fomukong & Dale, 1993) while vector sequence detection used labelled plasmid pGEM3Zf(+). For the pUS1800 series, the corresponding probes were the complete IS6110 sequence and the plasmid pUS1843. Results were recorded digitally by a Gel Documentation System (UVP) or ImageMaster (Pharmacia) and processed with CorelDraw and PhotoPaint.

RESULTS

Transposition from suicide and conditionally replicating plasmids in *M. smegmatis*

Transposition of IS6110 has previously been demonstrated in *M. smegmatis* (Fomukong & Dale, 1993), using a composite transposon on a suicide vector. In that

system, selection for kanamycin-resistant colonies identified events in which the *aph* gene has integrated into the chromosome, either through transposition or by non-homologous recombination.

To analyse the effects of context on transposition, we used two series of IS6110 composite transposon vectors, together with control plasmids lacking IS6110 or carrying only a single copy of IS6110 (Fig. 1). Transformation of the suicide vectors (pUS251–256) into *M. smegmatis* resulted in kanamycin-resistant transformants only if the kanamycin gene was integrated into the host chromosome.

The second series of vectors (pUS1800 series) was designed to obtain a larger number of transposition events and utilized the temperature-sensitive mycobacterial origin of replication from pCG59, which can replicate at 30 °C but is lost when cultures are incubated at 39 °C. Transposition from these plasmids was assessed using a papillation assay (Ghanekar *et al.*, 1999) as described in Methods.

Fig. 2 shows examples of the use of Southern blot analysis for assessing the occurrence of transposition events. With the suicide plasmid pUS252, five out of 11 transformants (Fig. 2a, lanes 1, 3–5 and 11) showed a variable junction fragment as well as the conserved internal band of 2.7 kb, consistent with transposition of the composite transposon; none of these lanes showed any hybridization with the vector sequence probe (results not shown). The remaining lanes appear to represent nonhomologous recombinants. Similarly, for

Table 1. Composite transposon structures and transposition in *M. smegmatis*

Suicide plasmid	Transposon structure			Kanamycin-resistant transformants	Transposition detected
	IS6110	<i>aph</i>	IS6110		
pUS251	←	←	→	Yes	No
pUS252	→	←	→	Yes	Yes
pUS253	←	←	←	Yes	No
pUS254	→	←	←	Yes	Yes
pUS255		←	→	Yes	No
pUS256		←	←	Yes	No
pGK		←		No	—
Conditional plasmid	Transposon structure			Papillation frequency	Transposition frequency
	IS6110	<i>aph</i>	IS6110		
pUS1846	→	←	←	4.2×10^{-5}	3.6×10^{-5}
pUS1847	←	←	←	2.3×10^{-5}	1.6×10^{-5}
pUS1849	→	←	→	2.3×10^{-5}	ND
pUS1845		←	→	ND ($< 2 \times 10^{-7}$)	—
pUS1843		←		ND ($< 2 \times 10^{-7}$)	—

ND, None detected.

the conditionally replicating vector pUS1846 transformants, 12 out of 14 clones (Fig. 2b, lanes 3–5, 7–9 and 11–16) examined showed variable junction fragments, a conserved internal band (2.2 kb) but no hybridization with vector sequences. This pattern was consistent with transposition. The remaining lanes gave patterns consistent with retention of the plasmid.

The results of this analysis, applied to each set of plasmids, are summarized in Table 1. With the suicide plasmids, transposition was only detected with pUS252 and pUS254. Although the nature of the assay and the small number of transformants available for testing make a quantitative comparison unreliable, the frequency of transposition from these plasmids can be tentatively estimated as 10^{-4} – 10^{-5} , by comparison with the transformation frequency of a shuttle plasmid. Amongst the conditionally replicating vectors, transposition was only detected for pUS1846 and pUS1847. The most striking feature is that for both sets of vectors effective transposition was not found to correlate with the relative orientation of the two copies of IS6110, but with the orientation of one of the copies relative to the plasmid backbone. This suggests that the mobility of IS6110 is influenced by its context.

Transposition from suicide plasmids in BCG

Transposition of IS6110 has not previously been experimentally demonstrated in BCG. Although BCG naturally contains IS6110 (IS987), the low copy number suggests that transposition of this element in BCG is a rare event. In order to test the ability of IS6110 to transpose in BCG we selected the suicide composite transposon plasmid pUS252 (which gave the highest proportion of transposition events in *M. smegmatis*) for transformation of BCG, in comparison with a plasmid (pUS256) carrying a single copy of the insertion sequence.

Transformation of BCG with pUS256 yielded less than 10 kanamycin-resistant colonies ($\mu\text{g DNA}$) $^{-1}$, which was similar to the frequency of kanamycin resistance found in a DNA-free control. Southern blot analysis was consistent with spontaneous mutation to kanamycin resistance (results not shown). Transformation with pUS252 produced kanamycin-resistant colonies at a frequency of about 15 transformants ($\mu\text{g DNA}$) $^{-1}$. In the same experiment, the shuttle plasmid pYUB12 transformed with a frequency of $>4 \times 10^4$ ($\mu\text{g DNA}$) $^{-1}$, indicating an apparent transposition frequency of $<3 \times 10^{-4}$. In subsequent experiments, using improved electroporation conditions (Wards & Collins, 1996), pUS252 transformation of BCG was obtained at higher frequencies [up to 100 colonies ($\mu\text{g DNA}$) $^{-1}$], enabling the use of this system for transposon mutagenesis of BCG (T. Jeyaseelaratnam & S. Wall, unpublished).

Southern blot analysis of representative BCG transformants (Fig. 3) is consistent with the occurrence of transposition in the majority of transformants, as demonstrated by hybridization of the IS6110 probe to

Fig. 3. Transposition from pUS252 in BCG. Southern blots of total DNA from kanamycin-resistant BCG colonies following transformation with pUS252, digested with *PvuII*, probed with the right-hand fragment of IS6110. A, Bands due to the resident copy of IS6110 (1.9 kb); B, the conserved fragment of the composite transposon (2.7 kb). Lanes: 1, BCG; 2–7, pUS252 transformants.

variable junction fragments in addition to the BCG-resident copy (1.9 kb) and the 2.7 kb internal fragment of the composite transposon. None of these lanes showed any hybridization to the vector-derived probe. Most transformants appeared to have acquired one additional copy of IS6110, but in some cases (such as lane 7 in Fig. 3) the strain appears to have acquired multiple copies. These extra events could have arisen by additional transposition events from the composite transposon, but could also be a consequence of mobilization of the resident copy of IS6110 by *trans* activation from the incoming copy (see below).

Activation of transposition from an external promoter

One explanation of the apparent context-sensitivity of transposition is that transcription of the coding region of the insertion sequence may require or be facilitated by an external promoter. The suicide plasmids described above are based on the pGEM3Zf(+) vector, which has a T7 promoter adjacent to the composite transposon. This is correctly oriented for transcription of the adjacent IS6110 in plasmid pUS253.

E. coli JM109(DE3), which carries an IPTG-inducible T7 RNA polymerase, was therefore transformed with pUS253. After induction with IPTG, colonies were

Fig. 4. Stimulation of transposition by activation of the T7 promoter. Lanes 1–18: total DNA from random colonies of *E. coli* JM109(DE3), carrying pUS253, after IPTG induction; *Pvu*II cut, Southern blotted and probed with IS6110 fragment.

picked at random and total DNA was tested by Southern blotting and hybridization with IS6110. Fig. 4 shows that three out of 18 colonies had additional insertion-sequence-hybridizing bands; on prolonged exposure, this ratio was even higher. Since there was no selection for transposition, this represents a very high degree of mobility. When we carried out the same procedure with pUS252, which has the insertion sequences in the opposite orientation, no transposition was detected. This confirms that transposition of IS6110 can be enhanced by the action of an external promoter.

Activation of transposition by transposase expressed *in trans*

The majority of *M. tuberculosis* isolates carry many copies of IS6110. Therefore, if the transposase is active *in trans*, activation of one copy of the element could result in mobilization of any of the copies, potentially amplifying the instability of the banding pattern. To test this hypothesis, the shuttle plasmid pUS267 (see Fig. 1) was constructed by cloning the *Bsp*EI fragment of IS6110 downstream from the promoter region of the *M. tuberculosis* *hsp60* gene, which has relatively strong promoter activity in mycobacteria. This fragment of IS6110 lacks most of the inverted repeat sequence at each end (and hence is not expected to be competent for transposition) but contains the complete ORFa and ORFb reading frames, including the putative ribosome-binding site for ORFa as identified by McAdam *et al.* (1990). The plasmid pUS267 was introduced into a strain of *M. smegmatis* (MS252.5) that already carried an integrated pair of copies of IS6110 arising from transformation with pUS252, as described above. The resulting transformants were analysed by Southern blotting. It should be noted that in this case, since the composite transposon was already present, selection (for hygromycin resistance) could only be made for transformation with the plasmid, not transposition; these were random transformants. All of the clones tested showed additional copies of the insertion se-

quence (together with those of the resident composite transposon and the shuttle plasmid) (Fig. 5a). Three of these bands were present, at varying intensities, in all of the clones, which suggests preferred insertion sites; some of the clones showed further bands as well. Since these clones had not been selected for transposition, these results indicate a high rate of transposition stimulated by the presence of the transposase on the shuttle plasmid. Probing with vector sequences gave results in all cases identical to those with pUS267 (results not shown), indicating the absence of plasmid rearrangements.

A similar experiment was performed to mobilize the resident copy of IS6110 in BCG. As with MS252.5, transformation with pUS267 does not allow selection for transposition. Southern blotting of transformants showed that in addition to the band due to the resident copy of the insertion sequence, all the clones tested exhibited additional bands hybridizing to the insertion sequence probe, suggesting a high rate of mobilization of the insertion sequence (Fig. 5b). The banding patterns were much more varied than those seen with transformation of *M. smegmatis* MS252.5. It can be seen from Fig. 5(b) that most of these clones had lost the 1 kb IS6110-hybridizing band characteristic of pUS267, raising the possibility that plasmid rearrangements could have contributed to the diverse banding pattern. Plasmid rescue experiments in *E. coli*, while confirming that these plasmids had lost part or all of the region encoding the transposase, showed no evidence of the high-molecular-mass (>4 kb) bands detected in Fig. 5(b).

One interpretation of these results is that the high frequency of transposition is damaging to the cells, probably because of lethal mutations arising from the high frequency of transposition of IS6110, thus providing a powerful selection pressure for deletion of the relevant region of the plasmid. That this did not occur in *M. smegmatis* MS252.5 could be ascribed to the apparently higher degree of insertion site preference (and hence a lower probability of mutation) that appeared to be exhibited in that host.

Fig. 5. Action of the transposase *in trans*. Southern blots of *Pvu*II-digested DNA, probed with the right-hand fragment of IS6110. (a) Lanes: 1–10, *M. smegmatis* MS252.5 (carrying an integrated composite transposon) transformed with pUS267; 11, MS252.5, untransformed; 12, *M. smegmatis* mc²155; 13, pUS252; 14, pUS267. A and B, Junction and internal bands from existing composite transposon in MS252.5; C and D, contributed by the shuttle plasmid pUS267. (b) Lanes: 1–14, BCG transformed with pUS267; 15, pUS267. A, Band due to the resident copy of IS6110 in BCG (1.9 kb); D, transposase fragment from pUS267.

DISCUSSION

The results obtained with sets of composite transposons, from both suicide and conditionally replicating plasmids, indicate that successful transposition of IS6110 in *M. smegmatis* is determined not by the relative orientation of the two copies but by the orientation of one copy with respect to flanking sequences in the vector plasmid. This context-sensitivity of transposition could be due to a requirement for an external promoter to drive transcription of the IS6110 sequences required for transposition. This is supported by the stimulation of

transposition seen when the adjacent T7 promoter in pUS253 is activated in *E. coli*. This suggests that in those strains of *M. tuberculosis* with only a single copy of IS6110, and similarly in *M. bovis* and BCG strains with only one copy, the presumed absence of transposition may be due to the element being present in a transcriptionally silent region of the chromosome. The same inference can be applied to other apparent 'hotspots' that occur typically but not exclusively in low-copy-number strains. The data reported here show that the composite transposon is indeed mobile in BCG; and the resident IS6110 copy in BCG can be mobilized *in trans*; both of these indicate that BCG is not inherently transpositionally incompetent. In addition, the data of Dale *et al.* (1998) show that the sequence of IS6110 (IS987) in BCG is identical to that in multi-copy strains of *M. tuberculosis*, and hence the element itself is not defective.

The context-sensitivity of transposition also implies that the frequency of transposition, and hence the rate of change of banding pattern, will vary between strains. It is therefore unreliable to deduce evolutionary or epidemiological distances between strains solely on the basis of degrees of similarity in their banding patterns. Furthermore, rare transposition events occurring in a strain with an otherwise stable banding pattern could result in a copy of the element being introduced into a more active site, which would be expected to result in a relatively sudden increase in the rate of variation. This effect could be amplified by the ability of the IS6110 transposase to mobilize other copies of the element *in trans*. Although in general IS6110 fingerprints are relatively stable (van Soolingen *et al.*, 1991; Yeh *et al.*, 1998), it remains possible that, in extreme cases, such a sudden increase in the rate of variation could result in epidemiologically connected strains undergoing sufficient variation for the banding patterns to be labelled as non-clustered.

ACKNOWLEDGEMENTS

K.G. was supported by a BBSRC studentship. This work was supported by the Commission of the European Communities under the BIOMED Programme.

REFERENCES

- Dale, J. W. (1995). Mobile genetic elements in mycobacteria. *Eur Respir J* 8 (suppl. 20), 633S–648S.
- Dale, J. W., Tang, T. H., Wall, S., Zainuddin, Z. F. & Plikaytis, B. (1998). Conservation of IS6110 sequence in strains of *Mycobacterium tuberculosis* with single and multiple copies. *Tuber Lung Dis* 78, 225–227.
- Dellagostin, O. A., Wall, S., Norman, E., O'Shaughnessy, T., Dale, J. W. & McFadden, J. (1993). Construction and use of integrative vectors to express foreign genes in mycobacteria. *Mol Microbiol* 10, 983–993.
- Fomukong, N. G. & Dale, J. W. (1993). Transpositional activity of IS986 in *Mycobacterium smegmatis*. *Gene* 130, 99–105.
- Fomukong, N. G., Dale, J. W., Osborn, T. W. & Grange, J. M. (1992). Use of gene probes based on the insertion sequence IS986

- to differentiate between BCG vaccine strains. *J Appl Bacteriol* 72, 126–133.
- Fomukong, N. G., Tang, T. H., Al-Maamary, S., Ibrahim, W. A., Ramayah, S., Yates, M., Zainuddin, Z. F. & Dale, J. W. (1994). Insertion sequence typing of *Mycobacterium tuberculosis*: characterization of a widespread sub-type with a single copy of IS6110. *Tuber Lung Dis* 75, 435–440.
- Ghanekar, K., McBride, A., Dellagostin, O. A., Mooney, R. & McFadden, J. (1999). Stimulation of transposition of the *Mycobacterium tuberculosis* insertion sequence IS6110 by exposure to a microaerobic environment. *Mol Microbiol* 33, 982–993.
- Guilhot, C., Gicquel, B. & Martín, C. (1992). Temperature-sensitive mutants of the *Mycobacterium* plasmid pAL5000. *FEMS Microbiol Lett* 98, 181–186.
- Hermans, P. W. M., van Soolingen, D., Dale, J. W., Schuitema, A. R. J., McAdam, R. A., Catty, D. & van Embden, J. D. A. (1990). Insertion element IS986 from *Mycobacterium tuberculosis*: a useful tool for diagnosis and epidemiology of tuberculosis. *J Clin Microbiol* 28, 2051–2058.
- Hermans, P. W. M., van Soolingen, D., Bik, E. M., De Haas, P. E. W., Dale, J. W. & van Embden, J. D. A. (1991). Insertion element IS987 from *Mycobacterium bovis* BCG is located in a hot-spot integration region for insertion elements in *Mycobacterium tuberculosis* complex strains. *Infect Immun* 59, 2695–2705.
- McAdam, R. A., Hermans, P. W. M., van Soolingen, D., Zainuddin, Z. F., Catty, D., van Embden, J. D. A. & Dale, J. W. (1990). Characterization of a *Mycobacterium tuberculosis* insertion sequence belonging to the IS3 family. *Mol Microbiol* 4, 1607–1613.
- Radford, A. J. & Hodgson, A. L. M. (1991). Construction and characterization of a *Mycobacterium-Escherichia coli* shuttle vector. *Plasmid* 25, 149–153.
- Snapper, S. B., Melton, R. E., Kieser, T., Mustafa, S. & Jacobs, W. R. (1990). Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. *Mol Microbiol* 4, 1911–1919.
- van Soolingen, D., Hermans, P. W. M., De Haas, P. E. W., Soll, D. R. & van Embden, J. D. A. (1991). Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. *J Clin Microbiol* 29, 2578–2586.
- Thierry, D., Cave, M. D., Eisenach, K. D., Crawford, J. T., Bates, J. H., Gicquel, B. & Guesdon, J. L. (1990). IS6110, an IS-like element of *Mycobacterium tuberculosis* complex. *Nucleic Acids Res* 18, 188.
- Wards, B. J. & Collins, D. M. (1996). Electroporation at elevated temperatures substantially improves transformation efficiency of slow-growing mycobacteria. *FEMS Microbiol Lett* 145, 101–105.
- Yeh, R. W., De Leon, P., Agasino, C. B., Hahn, J. A., Daley, C. L., Hopewell, P. C. & Small, P. M. (1998). Stability of *Mycobacterium tuberculosis* DNA genotypes. *J Infect Dis* 177, 1107–1111.
- Zainuddin, Z. F. & Dale, J. W. (1989). Polymorphic repetitive DNA sequences in *Mycobacterium tuberculosis* detected with a gene probe from a *Mycobacterium fortuitum* plasmid. *J Gen Microbiol* 135, 2347–2355.

Received 22 April 1999; revised 25 June 1999; accepted 20 July 1999.